

Morphological and Molecular Identification of *Longidorus euonymus* and *Helicotylenchus multicinctus* from the Rhizosphere of Grapevine and Banana in Greece

EMMANUEL A. TZORTZAKAKIS,¹ CAROLINA CANTALAPIEDRA-NAVARRETE,² PABLO CASTILLO,² JUAN E. PALOMARES-RIUS,²
AND ANTONIO ARCHIDONA-YUSTE²

Abstract: Plant-parasitic nematodes such as *Longidorus euonymus* and *Helicotylenchus multicinctus* are species widely distributed in central Europe as well as in Mediterranean area. In Greece, both species have been previously reported but no morphometrics or molecular data were available for these species. Nematode surveys in the rhizosphere of grapevines in Athens carried out in 2016 and 2017, yielded a *Longidorus* species identified as *Longidorus euonymus*. Similarly, a population of *Helicotylenchus multicinctus* was detected infecting banana roots from an outdoor crop in Tertsá, Crete. For both species, morphometrics and molecular data of Greek populations were provided, resulting in the first integrative identification of both nematode species based on morphometric and molecular markers, confirming the occurrence of these two nematodes in Greece as had been stated in earlier reports.

Key words: detection, needle and spiral nematodes, rDNA.

Longidorus euonymus Mali and Hooper, 1974 is a species widely distributed in central and south Europe found in a wide range of hosts including perennial, woody, annual and herbaceous plants (Oro et al., 2005). In Greece, the only report of this nematode is from artichoke crop at Marathon area near Athens, but no molecular data are available for this population (Roca et al., 1986). In spring 2016 and 2017, soil samples were collected from the rhizosphere of a grapevine at Athens. Nematodes were extracted from soil samples by wet sieving and decanting method (Cobb, 1918). A *Longidorus* species was found at a density of 1–3 specimens per 500 cm³ of soil.

Helicotylenchus multicinctus Cobb (Golden), 1956 is a parasite of bananas with a worldwide distribution (McSorley and Parrado, 1986). It has been found in the Mediterranean area in association with bananas in Cyprus, Lebanon, and Israel (Strich-Harari et al., 1966; Philis, 1971; Sikora and Schlosser, 1973). Furthermore, several populations of the nematode were found in Italy associated with other crops in uncultivated areas and dunes (Vovlas, 1983). Banana is cultivated in Crete (Greece) in greenhouses and outdoors, and this nematode has been previously reported in several cases (Vovlas et al., 1994; Tzortzakakis, 2008). However, morphometric characteristic or molecular data of the nematode were not provided. In autumn 2016, a *Helicotylenchus* species was found in infected roots from an outdoor banana crop at Tertsá, Crete. The nematode

was found at a density of five nematodes per gram of root, after incubating macerated root pieces in modified Baerman funnels.

Specimens to be observed under light microscopy were heat killed by adding hot 4% formaldehyde solution and processed to pure glycerin using De Grisse's (1969) method. Photographs were taken using a Zeiss III compound microscope with Nomarski differential interference contrast at up to ×1,000 magnification. Measurements and drawings were done using a drawing tube attached to the microscope. Specimens for molecular analysis were preserved in DESS (Yoder et al., 2006).

The Greek population of *L. euonymus* was characterized by a large body; lip region slightly expanded from the body contour, rounded and flattened frontally; amphidial fovea slightly asymmetrically bilobed, with aperture located at level with lip demarcation; vulva slightly anterior to midbody; and tail conical, bluntly with broadly rounded terminus with c' 1.3 (Table 1). These findings confirm the identity of the species and the intraspecific variability on several populations with different geographic origin and were within the range of the original description (Mali and Hooper, 1974) and other reported populations (Guesmi-Mzoughi et al., 2017). The Cretan population of *H. multicinctus* was characterized by a body arcuate to c-shaped when relaxed; lateral fields not areolated, with four incisures, about one-fourth of body width; lip region hemispherical, slightly offset, with three to five annuli; stylet well developed, 21 to 23 µm long, stylet knobs with outer margins directed forward (Table 1); tail slightly tapering, with a hemispherical annulated terminus, devoid of any ventral projection or mucro, with 10 to 12 annuli; and phasmids pore-like, two to four annuli anterior to anus level. These findings agreed with the original and previous descriptions of this nematode species (Siddiqi, 1973). A single individual of each species was used for DNA extraction. Primers and polymerase chain reaction conditions used in this research were

Received for publication June 29, 2017.

¹Department of Viticulture, Vegetable Crops, Floriculture and Plant Protection, Institute of Olive Tree, Subtropical Crops and Viticulture, N.AG.RE.F., Hellenic Agricultural Organization-DEMETER, 32A Kastorias Street, Mesa Katsabas, 71307 Heraklion, Crete, Greece.

²Institute for Sustainable Agriculture (IAS), CSIC, 14004 Córdoba, Spain.

The authors thank J. Martín Barbarroja and G. León Roperro from IAS-CSIC for the excellent technical assistance and Mr. A. Rizo from I.O.S.V.-NAGREF for collecting and sending the soil samples from the grapevine. Spanish authors acknowledge grant 201740E042, "Análisis de diversidad molecular, barcoding, y relaciones filogenéticas de nematodos fitoparásitos en cultivos mediterráneos" from Spanish National Research Council (CSIC).

E-mail: aarchidona@ias.csic.es.

This paper was edited by Zafar A. Handoo.

TABLE 1. Morphometrics of *Longidorus euonymus* (Mali and Hooper, 1974) and *Helicotylenchus multincinctus* Cobb (Golden), 1956 from Greece. (All measurements in μm and in the format: mean \pm SD (range).*)

	<i>L. euonymus</i>	<i>H. multincinctus</i>
Locality (code sample)	Athens (sample 44)	Tertsa (sample 1)
Host	Grapevine	Banana crop
Character/Ratio	Females	Females
<i>n</i>	3	4
L	7,379 \pm 161 (7,205–7,523)	570 \pm 65.4 (508–640)
a	170.5 \pm 9.0 (163.8–180.7)	21.5 \pm 1.8 (19.2–23.5)
b	17.8 \pm 1.8 (15.7–18.9)	-
b'	-	5.4 \pm 0.9 (4.3–6.5)
C	191.8 \pm 7.8 (184.7–200.2)	51.7 \pm 4.0 (46.2–55.5)
c'	1.3 \pm 0.1 (1.2–1.4)	1.0 \pm 0.1 (0.9–1.1)
V	46.0 \pm 1.0 (45.0–47.0)	65.3 \pm 1.5 (64.0–67.0)
Odontostyle length	83.0 \pm 3.6 (80.0–87.0)	-
Odontophore length	61.3 \pm 4.0 (59.0–66.0)	-
Stylet length	-	22.3 \pm 1.0 (21.0–23.0)
DGO	-	8.8 \pm 0.6 (8.0–9.5)
O (%)	-	39.3 \pm 1.4 (38.1–41.3)
Lip region width	13.0 \pm 1.0 (12.0–14.0)	-
Oral aperture-guiding ring	26.5 \pm 2.5 (24.0–29.0)	-
Anterior end to excretory pore	-	89.3 \pm 4.8 (84.0–95.0)
Pharynx length	-	139.0 \pm 10.0 (129.0–152.0)
Tail length	38.5 \pm 1.3 (37.0–39.5)	11.0 \pm 0.8 (10.0–12.0)
Tail annuli	-	8.0 \pm 1.4 (7.0–10.0)
Hyaline region length	9.8 \pm 1.1 (9.0–10.5)	-

* Abbreviations are defined in Jairajpuri and Ahmad (1992) and Siddiqi (2000).

specified in Vrain et al. (1992) and Archidona-Yuste et al. (2016). Single amplicons of 900 and 1,100 bp were obtained and sequenced for D2–D3 and ITS ribosomal DNA regions, respectively. Sequence alignments for D2–D3 (MF401444) of *L. euonymus* from Greece showed 99% similarity to other sequences of *L. euonymus* deposited in GenBank from Hungary, Russia, and Tunisia, differing in 2 to 9 bp and 0 indels (AY601573, KF242331 to KF242333, and KF062667; He et al., 2005; Subbotin et al., 2014; Guesmi-Mzoughi et al., 2017). Sequence alignments of ITS1 from the Greek population (MF401445) showed 99% similarity to other sequences of *L. euonymus* deposited in GenBank from Tunisia, differing in 6 to 7 bp and one indels (KF062691 to KF062692; Guesmi-Mzoughi et al., 2017). Sequence alignments for D2–D3 (MF401446) of *H. multincinctus* from Crete showed 100% to 99% similarity to other sequences of *H. multincinctus* deposited in GenBank from Florida and China, differing in 0 to 3 bp and zero indels (HM014292, KF443214; Subbotin et al., 2011). Sequence alignments of ITS from the Cretan population (MF401447) showed from 99% to 97% similarity to other sequences of *H. multincinctus* deposited in GenBank from Malaysia and Fujian (China), differing in 4 to 35 bp and zero to eight indels (FJ460169 to FJ460173, and KF443216), respectively. Morphology, morphometry, and molecular data obtained from these samples were consistent with *L. euonymus* and *H. multincinctus* identification. This report consists the first integrated identification of both nematode species based on morphometric and molecular characteristics and confirms the occurrence of

these two nematodes in Greece as had been stated in earlier reports.

LITERATURE CITED

- Archidona-Yuste, A., Navas-Cortés, J. A., Cantalapiedra-Navarrete, C., Palomares-Rius, J. E., and Castillo, P. 2016. Unravelling the biodiversity and molecular phylogeny of needle nematodes of the genus *Longidorus* (Nematoda: Longidoridae) in olive and a description of six new species. *PLoS One* 11:e0147689.
- Cobb, N. A. 1918. Estimating the nema population of soil. Agricultural Technology Circular, Bureau of Plant Industry, United States Department of Agriculture.
- De Grisse, A. T. 1969. Redescription ou modifications de quelques techniques utilisées dans l'étude des nématodes phytoparasitaires. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent* 34:351–369.
- Guesmi-Mzoughi, I., Archidona-Yuste, A., Cantalapiedra-Navarrete, C., Palomares-Rius, J. E., Regaieg, H., Horrigue-Raouani, N., and Castillo, P. 2017. Integrative identification and molecular phylogeny of dagger and needle nematodes associated with cultivated olive in Tunisia. *European Journal of Plant Pathology* 147:389–414.
- He, Y., Subbotin, S., Rubtsova, T. V., Lamberti, F., Brown, D. J. F., and Moens, M. 2005. A molecular phylogenetic approach to Longidoridae (Nematoda: Dorylaimida). *Nematology* 7:111–124.
- Jairajpuri, M. S., and Ahmad, W. 1992. *Dorylaimida: Freelifving, predaceous and plant-parasitic nematodes*. New Delhi, India: Oxford & IBH Publishing.
- Mali, V. R., and Hooper, D. J. 1974. Observations on *Longidorus euonymus* n. sp. and *Xiphinema vuittenezi* Luc et al., 1964 (Nematoda: Dorylaimida) associated with spindle trees infected with euonymus mosaic virus in Czechoslovakia. *Nematologica* 19:459–467.
- McSorley, R., and Parrado, J. L. 1986. *Helicotylenchus multincinctus* on bananas: An international problem. *Nematropica* 16:73–90.
- Oro, V., Hubschen, J., Karanastasi, E., Krnjajic, D., Brown, D. J. F., and Neilson, R. 2005. Inter-population variability of *Longidorus euonymus* Mali and Hooper, 1974 (Nematoda, Dorylaimida) and comment upon

- the number of juvenile developmental stages. *Helminthologia* 42:155–165.
- Philis, J. 1971. Control of root-knot and spiral nematodes on bananas in Cyprus. *Plant Disease Reporter* 55:707–710.
- Roca, F., Rana, L. G., and Kyriakopoulou, P. 1986. Studies on Longidoridae (Nematoda, Dorylaimida) and raspberry ringspot virus spread in some artichoke fields in Greece. *Nematologia mediterranea* 14:251–256.
- Siddiqi, M. R. 1973. *Helicotylenchus multicinctus*. C.I.H. Descriptions of plant-parasitic nematodes. Set. 2, no. 23, 3 pp.
- Siddiqi, M. R. 2000. *Tylenchida Parasites of Plants and Insects*, 2nd ed. Wallingford, UK: CABI Publishing.
- Sikora, R., and Schlosser, E. 1973. Nematodes and fungi associated with root systems of bananas in a state of decline in Lebanon. *Plant Disease Reporter* 57:615–618.
- Strich-Harari, D., Minz, G., and Peled, A. 1966. The spread of spiral nematodes in banana roots and their control. *Israel Journal of Agricultural Research* 16:89–94.
- Subbotin, S. A., Inserra, R. N., Marais, M., Mullin, P., Powers, T. O., Roberts, P. A., Van den Berg, E., Yeates, G. W., and Baldwin, G. 2011. Diversity and phylogenetic within the spiral nematodes of *Helicotylenchus* Steiner, 1945 (Tylenchida: Hoplolaimidae) as inferred from analysis of the D2–D3 expansion segments of 28S rRNA gene sequences. *Nematology* 13:333–345.
- Subbotin, S. A., Rogozhin, E., and Chizhov, V. 2014. Molecular characterisation and diagnostics of some *Longidorus* species (Nematoda: Longidoridae) from Russia and other countries using rRNA genes. *European Journal of Plant Pathology* 138:377–390.
- Tzortzakakis, E. A. 2008. Plant parasitic nematodes associated with banana crop in Crete, Greece. *Acta Agriculturae Slovenica* 91:97–101.
- Vovlas, N. 1983. Morphology of a local population of *Helicotylenchus multicinctus* from southern Italy. *Review de Nematologie* 6:327–329.
- Vovlas, N., Avgelis, A., Goumas, D., and Frisulo, S. 1994. A survey of banana diseases in sucker propagated plantations in Crete. *Nematologia Mediterranea* 22:101–107.
- Vrain, T. C., Wakarchuk, D. A., Levesque, A. C., and Hamilton, R. I. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15:563–573.
- Yoder, M., Tandingan de Ley, I., King, I. W., Mundo-Ocampo, M., Mann, J., Blaxter, M., Poiras, L., and De Ley, P. 2006. DESS: A versatile solution for preserving morphology and extractable DNA of nematodes. *Nematology* 8:367–376.